Method for amplifying at least one specific nucleotide sequence, and primers used

The present invention relates to a novel method for amplifying at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture. It also relates to primers which enable such an amplification.

The state of the art describes methods for amplifying nucleotide sequences using primers specific for these sequences to be amplified. Thus, it is possible to amplify a gene, or a family of genes, in a preparation of nucleic acids. Many techniques use oligonucleotides complementary to the target sequence which serve as primers for elongation by a polymerase.

For the amplification of DNAs, there exists the PCR (Polymerase Chain Reaction), as described in Patents US-A-4,683,195, US-A-4,683,202 and US-A-4,800,159, the LCR (Ligase Chain Reaction), set out for example in Patent Application EP-A-0,201,184, or the RCR (Repair Chain Reaction), described in Patent Application WO-A-90/01069.

For the amplification of RNAs, several techniques have also been described in various documents. These techniques are as follows:

- 3SR (Self Sustained Sequence Replication) with Patent Application WO-A-90/06995,

- NASBA (Nucleic Acid Sequence-Based Amplification) with Patent Application WO-A-91/02818,

- SPSR (Single Primer Sequence Replication) with Patent US-A-5,194,370, and

- TMA (Transcription Mediated Amplification) with Patent US-A-5,399,491.

However, these techniques impose a rigorous choice of amplification primers. Specifically, primers which are relatively nonspecific for the sequence of interest will allow the amplification of many related sequences to which they are bound. The amplicon corresponding to the sequence of interest will

therefore be diluted in a mixture of amplicons, which will not facilitate the use of this amplification product. Under these conditions, it is then essential to rigorously choose a region of the nucleotide sequence which is sufficiently specific to allow a complementary primer which is just as specific to be produced. However, other problems may surface when choosing the sequence to be used for the hybridization of the primer. Specifically, the region which is really specific is sometimes unique and is located on the inner part of the sequence of interest. Choosing to hybridize a primer to such a region implies the production of only a varyingly significant fraction of said sequence of interest. There is therefore loss of information. In addition, the production of a primer specific for the nucleotide sequence of interest brings considerably more difficulty and work.

With the present invention, the risks of obtaining truncated amplification products and the difficulties in obtaining primers specific for the nucleotide sequence to be amplified are eliminated, since it is possible to amplify specifically the nucleotide sequence of interest under conventional hybridization conditions.

To do this, two types of complementary primer are used; firstly, a type of primer which hybridizes indifferently to all related nucleotide sequences and, secondly, a type of primer or [sic] each primer hybridizes to only one of these related sequences. The first, which are nonspecific, will be used as primers for the elongation, the second, which are specific for nucleotide sequences related to the sequence of interest, will be used as primers for blocking the elongation of some of these related nucleotide sequences.

When a mixture of nonspecific and specific primers is used, depending on the choice of the type of specific sequence used, the elongation of certain

nonspecific sequences can be prevented. It is then possible to select the amplicons which are obtained.

Thus, the amplification of certain related sequences whose amplification is not desired can be blocked by adding complementary sequences specific for these related sequences, these specific complementary sequences acting as blocking primers. Thus, the sequence(s) of interest, which will be amplified selectively, is (are) isolated. A single amplicon is therefore obtained for each sequence of interest for which no blocking primer has been added.

To this effect, the present invention relates to a method for amplifying at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture, the reaction mixture consisting of at least one nucleic acid comprising at least two related nucleotide sequences and/or of at least two nucleic acids, each comprising at least one related nucleotide sequence, the method using at least one type of amplification primer capable of hybridizing with the nucleic acid so as to allow the amplification of the related nucleotide sequences, characterized in that it consists in adding, to the reaction mixture, at least one sequence, acting as a blocking primer, which is capable:

- of hybridizing to at least one nucleotide sequence, which is not the specific nucleotide sequence(s) to be amplified, and
- of preventing, at the level of this nucleotide sequence, the elongation of the amplification primer.

Preferably, the blocking primer(s) is (are) capable of hybridizing to the, or to all the, nucleotide sequences which are not the specific nucleotide sequence(s) to be amplified.

Firstly, in the case of a blocking primer used in an amplification method, as described above, each blocking primer is an oligonucleotide based on modified nucleotides and/or ribonucleotides and/or deoxyribonucleotides, such as PNAs or thiophosphate nucleotides.

Secondly, in the case of a blocking primer used in an amplification method, as described above, each blocking primer comprises at least one element which prevents the amplification.

Thus, the element which prevents the amplification is located at the 3′ end of the blocking primer and does not allow its elongation.

In addition, another element which prevents the amplification is located at the 5′ end of the blocking primer and acts as a protective element.

Each element which prevents the amplification consists:

- either of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide not hybridizing to the nucleic acid,
- or of a molecule other than a nucleotide or than a modified nucleotide.

In this case, the element consists of at least five, in particular at least ten, and preferably at least fifteen, nucleotides or modified nucleotides or a mixture of nucleotide(s) and modified nucleotide(s).

According to a first embodiment, and when the element consists of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide does not hybridize to the nucleic acid and the element is sufficiently long to allow the formation of a loop and of hybridization between the nucleotides and/or modified nucleotides which constitute this loop.

According to a second embodiment, and when the element consists of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide does not hybridize to the nucleic acid and the element consists of a "tail" of polynucleotides and/or of

modified polynucleotides, which comprises all the same bases.

In the case of a blocking primer which is used in an amplification method, and which comprises an element which does not allow the elongation, the element is substituted for the hydrogen atom of the hydroxyl group or for the hydroxyl group, placed at the 3′ position of the ribose, itself located at the 3′ end of the nucleic acid.

In the case of a blocking primer which is used in an amplification method, and which also comprises a protective element, the element is:

- substituted for the phosphate placed at the 5′ position of the ribose, itself located at the 5′ end of the nucleic acid, or
- grafted onto the phosphate placed at the 5′ position of the ribose, itself located at the 5′ end of the nucleic acid.

The attached figures are given by way of explanatory example and are not limiting in nature.

Figure 1 represents a schematic view of the principle of an amplification of a strand of nucleic acid and of its complementary strand, using two primers; in this case, which is the simplest, there is one primer per strand.

Figure 2 represents a schematic view of the principle of an amplification according to Figure 1, but using the technique set out by the present invention.

Figure 3 represents the various substitutions which can be carried out on the nucleotides of the blocking primer, in which:

- R1 is an element which is at the 3′ end of the blocking primer and which prevents any elongation during the amplification,
- R2 is an element which can be on at least one of the 2′ positions of the ribose of a nucleotide of the blocking primer, and which reinforces the stability of the blocking primer/nucleic acid duplex, and

- R3 is an element which is at the 5′ end of the blocking primer and which acts as a protective element.

Figure 4 represents the positioning of the R1 element at the 3′ end of the blocking primer when said primer is hybridized to the nucleic acid.

Figure 5 represents the positioning of the R3 element at the 5′ end of the blocking primer when said primer is hybridized to the nucleic acid.

Figure 6 represents the blocking primer/nucleic acid duplex, in which X represents a nucleotide of the blocking primer comprising, in position on the ribose of this nucleotide, the R2 element which reinforces the stability of the duplex.

Figure 7 represents various structures which, by being added at the 3′ position of the blocking primer, prevents [sic] any elongation during the amplification.

Figure 8 represents various structures which, by being added at the 5′ position of the blocking primer, in addition to the modifications at the 3′ position, as represented in Figure 3, act as a protective element by preventing the degradation or ejection of the blocking primer during the amplification.

Figure 9 represents the electrophoregram corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1*1301 and HLA-DRB3*01 lymphoblastoid line, observed for the region encoding amino acids 56 to 65 (according to HLA official nomenclature) of the HLA-DRB genes without using a blocking primer.

Figure 10 represents the electrophoregram corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1*1301 and HLA-DRB3*01 lymphoblastoid line, observed for the region encoding amino acids 56 to 65 (according to HLA official nomenclature) of the HLA-DRB genes using a 5′-phosphate/3′-C_6-NH_2 oligonucleotide which inhibits the amplification of the HLA-DRB3 gene.

Figure 11 represents the electrophoregram corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1*0901 and HLA-DRB4*01 lymphoblastoid line, observed for the region encoding amino acids 29 to 47 (according to HLA official nomenclature) of the HLA-DRB genes without using a blocking primer.

Figure 12 represents the electrophoregram corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1*0901 and HLA-DRB4*01 lymphoblastoid line, observed for the region encoding amino acids 29 to 47 (according to HLA official nomenclature) of the HLA-DRB genes using a 5'-acridine/3'-H oligonucleotide which inhibits the amplification of the HLA-DRB4 gene.

Figure 13 represents a schematic view of the principle of a selective amplification of a gene in a family of related genes located on the same chromosome.

The present invention therefore relates to, inter alia, the use of oligonucleotide primers modified at their ends, for selectively amplifying genes.

The invention also relates to a method using modified oligonucleotide primers, for selectively amplifying certain genes present in a set of related genes.

The analysis of genes of interest is facilitated by the use of gene amplification techniques which make it possible to prepare, from a biological sample, amounts of specific material which can be easily analyzed using the conventional techniques of molecular biology. Thus, the use of oligonucleotide primers bordering a gene region leads to the production of a mixture of nucleotide molecules which is considerably enriched in the molecule of interest, which molecule then becomes easily detectable using electrophoretic analysis techniques or molecular hybridization techniques. The effectiveness of this approach lies in the use of oligonucleotide primers specific for the gene regions to be analyzed. These

primers must, therefore, be oligonucleotide sequences capable of hybridizing selectively with the nucleic acid sequences of interest which are present in the sample.

The analysis of genes which are members of families of structurally close genes can, however, sometimes be delicate. The search for nucleotide regions which are unique for a given gene makes it possible to achieve the desired specificity, but this approach can sometimes turn out to be difficult, or even impossible.

The present invention consists, therefore, in combining the use of oligonucleotide primers specific for the amplification of a limited set of structurally close genes, and of modified oligonucleotide primers capable of specifically blocking the unwanted genes. In fact, each type of these modified primers corresponds to a single type of unwanted gene.

This strategy makes it possible to simplify the analysis of genes in a mixture, by determining their nucleotide sequence (by sequencing in a gel, for example, or by multiple hybridization sequencing - DNA chip -) or by analyzing mutations.

The invention claims the use of mixtures of nucleotide primers enabling the effective amplification of corresponding nucleic acid regions, and of blocking nucleotide primers, which overlap or are located downstream (with respect to the nucleotide primer enabling the amplification), consisting of oligonucleotides which cannot serve as initiator sequences for the elongation, and therefore the amplification, of the downstream sequences.

Thus, for an unwanted gene or allele, the nonblocking primer and the blocking primer hybridize to the same strand for a given primer polarity (5′ primers upstream, or 3′ primers downstream, of the region to be analyzed). The nonblocking primer, if it manages to hybridize to the strand to be amplified, cannot generate amplicons beyond the region corresponding to

the hybridization site of the blocking primer, making the amplification corresponding to the nonblocking primer ineffective. This principle, illustrated in Figures 1, 2 and 13, concerns the functioning of the blocking primers and of the nonblocking primers.

According to Figure 1, an amplification is carried out with nonblocking primers P1 and P2. In an entirely conventional way, the extension of the P1 and P2 primers proceeds, and multiple amplicons A are obtained.

According to Figure 2, Figure 1 is repeated exactly, since it involves an amplification with nonblocking primers P1 and P2. However, on the complementary strand, and downstream of the progression of the P1 primer elongation, a sequence, acting as a blocking primer P1b, is added which is capable of hybridizing to the complementary strand and of preventing the amplification at the level of this strand. In this scenario, no amplicon will be produced.

According to Figure 13, Figures 1 and 2 are repeated exactly since it involves a selective amplification of the G_2 gene by blocking the related genes G1 [sic] and G3 [sic] using specific blocking primers and nonspecific amplification primers.

The invention claims the use of blocking primers comprising modified nucleotides. This principle is illustrated in Figures 3 to 6.

According to Figure 3, the nucleotide can be modified on the 2′ or 3′ positions of the ribose at the 3′ end of the oligonucleotide, and on the 5′ position of the ribose at the 5′ end of said oligonucleotide.

According to Figure 4, the R1 group replaces, at the 3′ position of the ribose, the hydroxyl and makes it possible to prevent the elongation of the 3′ end of the primer by the polymerase, when the nucleic acid/blocking primer duplex is formed.

According to Figure 5, the R3 group replaces, at the 5′ position of the ribose, the phosphate and makes it possible to protect the blocking primer

against degradation of the 5′ end and/or against being displaced, during the elongation of the amplification primer.

According to Figure 6, the nucleic acid/blocking primer duplex can be reinforced by substitution of the hydroxyl or of the hydrogen at the 2′ position of the ribose, this substitution possibly taking place on several nucleotides of the blocking primer. The R2 group can be, for example, a 2′-O-methyl radical, which stabilizes the DNA/RNA duplexes by creating a hydrophobic-type interaction.

There are many uses for the claimed strategy each time a mixture of related sequences is to be analyzed: human or animal genetics, analyses of infectious agents (viruses, bacteria, parasites, etc.).

By way of example, uses in the domain of HLA (for Human Leukocyte Antigens) typing are described below.

Thus, the major histocompatibility complex (MHC) comprises a set of genes which are located on chromosome 6 in humans, and which are involved in regulating the immune response (Bodmer et al. *Nomenclature for factors of the HLA System, 1996,* Tissue Antigens, 1997, 49, 297-321). A very great polymorphism is observed for these genes, and an entirely specific set of versions (or alleles) of each of these genes is observed for each individual. It is important to note that any individual has two alleles of each gene, one inherited from the mother, the other from the father.

In this set of MHC genes, more commonly named "HLA genes", some are henceforth well known, both their nucleic acid sequence and the functions of the corresponding proteins. They are essentially HLA genes termed class I HLA genes (HLA-A, HLA-B, HLA-Cw) and HLA genes termed class II HLA genes (HLA-DR, HLA-DQ and HLA-DP). These genes participate in the regulation of the immune response at the level of the surveillance of the integrity of self, with various consequences in the

medical domain. A first use relates to the field of organ or bone marrow grafts, and many studies have demonstrated the importance of optimum pairing, between the organ donor and the recipient, for the HLA genes therefore involved in the histocompatibility. A second use relates to the study of the susceptibility of each individual to develop certain pathologies induced by infectious agents (viruses, bacteria, parasites) or by other mechanisms which are still poorly understood (in the case of autoimmune diseases, for example). The HLA genes participate then in the development of the very great diversity of the immune response, observed in each individual, for a given ethnic group. Finally, the determination of the alleles of the HLA genes allows a precise characterization or identification of any individual, constituting a third domain of use of HLA typing.

One of the main difficulties of HLA typing lies in the structural homology observed for these genes, since they have evolved from common ancestors. It results therefrom that the genes of interest are to be analyzed within a set of close functional genes, or of nonfunctional genes (pseudogenes). It is therefore essential to control as well as possible the targeting of the analysis of the nucleic acid sequences, by optimizing the techniques for amplification of the regions to be sequenced.

For example, HLA-A typing is based on the selective analysis of the two alleles of the HLA-A gene observed in an individual, while avoiding the analysis of the structurally close genes HLA-B and HLA-Cw. It is therefore essential to be able to specifically amplify the related regions observed for the HLA-A gene, using nucleotide primers capable of hybridizing only with the regions targeted on this gene.

Another example relates to HLA-DR typing, in which the analysis of polymorphism concerns only the HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 genes, which correspond to the genes encoding the polypeptide chains

constituting the functional proteins expressed at the surface of the cells, while avoiding the co-amplification and analysis of the HLA-DRB2, HLA-DRB6, HLA-DRB7, HLA-DRB8 and HLA-DRB9 pseudogenes. The HLA-DR-typing example illustrates the great complexity of the mixture of nucleic acid sequences to be identified which are encountered for a given sample, and the presence of two alleles for each of the genes further increases the difficulty, sometimes making the interpretation of the results very delicate (typing ambiguities).

Taking HLA-DR typing as an example, it may prove that simplifying the analysis may be very beneficial, by restricting it only to the analysis of the HLA-DRB1 gene (with its two alleles for each individual), if the molecular analysis techniques employed use the determination of signal intensity (intensity of fluorescence for molecules of increasing sizes for sequencing) or the interpretation of oligoprobe hybridization reactivity profiles, for example. This objective may be attained by selecting nucleotide primers for HAL-DRB1-specific amplification, but this approach is not always possible because of the nucleic acid sequences observed for the various alleles of the HLA-DRB1 gene and of the sequences of the other HLA-DRB genes, which share great homology. One alternative consists of the present invention, and is based on the use of a mixture of primers which are specific for the HLA-DRB genes but nonspecific for the HLA-DRB1 gene, and of blocking primers which are specific for the HLA-DRB3, HLA-DRB4 and HLA-DRB5 genes. Selective amplification of the HLA-DRB1 genes therefore results therefrom, allowing the two HLA-DRB1 alleles observed for a given individual to be determined more easily.

The nucleotide primers are synthesized according to conventional methods, such as those using solid phase synthesis for example, and, unless otherwise stated, comprise an -OH residue in the 3′

region on the sugar (3'-OH), which allows their elongation during the amplification step. They are essentially oligonucleotides with a length of between 10- and 30-mer, according to the uses, this depending on the nucleic acid sequences considered.

The blocking nucleotide primers are prepared according to the methods mentioned above and contain a functional group, which inhibits the elongation, located at the 3' end of the oligonucleotide. The object of this blocking functional group is to prevent the addition, by the DNA polymerase, of the next base according to the information read on the complementary sequence. By way of example, this 3' blocking functional group may be a phosphate-alkylamine (C_6-NH_2), phosphate or dabcyl group; with regard to this subject, see Figure 7. These groups protect the hydroxyl functional group (3'-OH), and thus block its reactivity during the polynucleotide polymerization catalyzed by the DNA polymerase. The blocking of the enzymatic polymerization can also be obtained by dehydroxylation of the 3' position. In fact, primers containing 3'-H, 2'-OH ends can be obtained using suitable reagents and the technique of oligonucleotide assembly on a solid support.

If the use of a blocking primer which does not overlap with the nonblocking primer must be envisaged, it may be advantageous to also protect the 5' end of the blocking primer, so that this primer is neither degraded by the exonuclease activity of the polymerase, nor displaced during the elongation of the nonblocking primer located further upstream (further to the 5' site) of the region to be amplified. For this, various modifications may make it possible to maintain the integrity of the blocking primer hybridized to the nucleic acid sequence to be inactivated. Several possibilities may be mentioned by way of examples: the acridine nucleus, dimethoxytrityl (DMT), the thiophosphate group and a "stem-loop" additional

sequence. Such modifications are correctly represented in Figure 8.

The acridine nucleus is a powerful intercalating agent; it thus confers very great stability on the primer/target sequence duplex and avoids the displacement of the primer. The dimethoxytrityl (DMT) used as a protective group for the 5'-hydroxy end, the thiophosphate group used in the antisense strategy, and an additional sequence capable of forming a secondary "stem-loop" structure, protect the primer against possible degradation by exonuclease activity.

3' modification	$-C_6-NH_2$ -phosphate -H -dabcyl
5' modification	-acridine -DMT -thiophosphate -"stem-loop" structure

Table 1: Modifications of the 3' (and) 5' ends of the blocking primers

The principle of using mixtures of nonblocking primers and of blocking primers can be used for the 3' end (downstream), or for the 3' end and for the 5' end (upstream of the region to be analyzed), depending on the characteristics of the nucleic acid sequences or depending on the complexity of the genes of the region to be analyzed.

This approach of amplification using blocking primers can also be used for strategies of simple amplification corresponding to a mixture of primers capable of hybridizing to the same nucleic acid sequence to be analyzed, or in multiplex corresponding to several mixtures of primers capable of hybridizing, during the same amplification reaction, to various

nucleic acid sequences to be analyzed (various loci, various genes or various regions of a gene).

Example of synthesis of 3′- and 5′-blocked oligonucleotide primers

The blocked oligonucleotide primers were synthesized using an Expidite machine (Perseptive Biosystems) according to the automatic phosphoramidite method in accordance with the protocol proposed by the constructor. The phosphoramidite reagents required for the introduction of the 3′ and 5′ modifications were obtained from Glenn Research.

The oligonucleotide purification is carried out by reverse phase HPLC (semipreparative 10 mm × 25 cm Beckman ODS column; C18; 5 μm in porosity; eluent: 30 min gradient of 10 to 30% of acetonitrile in a mixture with an aqueous solution of 0.1 M triethylammonium acetate at pH 7). The fractions containing the oligonucleotide were collected and dried. The various oligonucleotides were then taken up in pure water and quantified by measuring the UV absorption.

To illustrate the principle of the present invention, two examples of use in the HLA domain are described below.

Example 1: Specific blocking of the amplification of the HLA-DRB3 gene

Several HLA-DRB genes can encode an HLA-DRβ polypeptide chain: HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5. The organization of this set of functional genes transmitted in hereditary fashion varies according to individuals, who thus exhibit various conserved haplotypes. If the presence of an HLA-DRB1 gene is always observed, the presence of one or two other genes (HLA-DRB3, HLA-DRB4, HLA-DRB5) is facultative, depending on the HLA-DRB1 allele carried by the same chromosome. Moreover, because of the presence of the two haplotypes (one inherited from the mother, the other from the father), the complexity of the mixture of the HLA-DRB sequences to be analyzed is

very variable. The analysis of the main information associated with the HLA-DRB1 alleles can therefore be difficult to interpret depending on whether the other HLA-DRB genes, such as for example HLA-DRB3, are present or not. It may therefore be advantageous to be able to amplify all the possible HLA-DRB1 alleles (184 listed in the 1997 nomenclature, *Nomenclature for factors of the HLA system, 1996, Tissue Antigens, 49, 3-II, March 1997*) without amplifying the HLA-DRB3 alleles possibly present (1 or 2 possible alleles among the 11 alleles listed in the 1997 nomenclature).

In order to illustrate the specific inhibition of the amplification of the DRB3 gene, two examples are described below, the first using an oligonucleotide comprising a 3′ end modified by incorporating an amine-containing arm (oligonucleotide 5858, SEQ ID 3) and the second using an oligonucleotide comprising a modified 3′ end comprising an -H, and a modified 5′ end comprising an acridine (oligonucleotide 5967, SEQ ID 4).

The DNAs were extracted according to conventional techniques of cell lysis and of proteinase K digestion, and then purified by ethanol precipitation, after phenol extraction. The DNAs solutions (concentration adjusted to 100 ng/μl in H_2O), are conserved at 2-8°C.

The general conditions used for amplification were as follows:

- 10X buffer	:	10 μl
- Generic 5′ primer (5867, SEQ ID NO 1)(10 μM)(0.1 μM final)	:	1 μl
- Blocking 5′ primer (10 μM)(0 to 1.2 μM final)	:	0 to 12 μl
- Generic 3′ primer (P2, SEQ ID NO 2)(10 μM)(0.1 μM final)	:	1 μl
- dNTP (20 mM) (0.2 mM final)	:	1 μl
- Taq polymerase (5 IU/μl)(1.5 U)	:	0.3 μl
- DNA (100 ng/μl)(100 ng)	:	1 μl
- H_2O (QS)	:	100 μl

The characteristics of the amplification program used with a Perkin Elmer GeneAmp 9600 machine, were as follows:

2 min at 95°C (1 cycle)

30 sec at 95°C + 30 sec at 55°C + 30 sec at 72°C (32 cycles)

7 min at 72°C (1 cycle)

The amplification products obtained were controlled by analyzing an aliquot portion (5 μl) by agarose gel electrophoresis and then staining with ethidium bromide. After this control, the amplicons prepared were analyzed with the bioMérieux oligodetection HLA-DR-typing kit (Ref. 74 500). This test makes it possible to determine the HLA-DR typing using a reverse hybridization technique in microplates, by detecting and analyzing the HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 alleles (PCT/FR92/00702).

DRB3 blocking with 5′-phosphate/3′-C_6-NH_2 oligo:

DNA: OMW line (ECCAC 9058), DRB1*1301, DRB3*01

5′ generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1 μM final

3′ generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1 μM final

5′ blocking HLA-DRB3 primer (oligonucleotide 5858, SEQ ID NO 3): 0, 0.3, 0.6, 0.9, 1.2 μM final

<u>Microplate hybridization:</u>

The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 2 below.

Final conc. of blocking primer 5858 (μM)	Probe 13	Probe 3+6	Probe 52a
0	> 2500	723	928
0.3	2109	578	101
0.6	1901	563	29
0.9	1759	374	31
1.2	1719	503	12
0.6/0 ratio	0.76	0.78	0.03

Table 2: Signals for hybridization of the blocking primer 5858 to various target sequences as a function of its concentration

The calculation of the ratio of the value read for 0.6 μM with respect to the value read without blocking, makes it possible to assess the inhibition of the amplification of the DRB3 gene.

Probes 13 and 3+6 are specific for the DRB1 gene, and probe 52a is specific for the DRB3 gene. The addition of oligonucleotide 5858 during amplification inhibits the amplification of the DRB3 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB3 oligonucleotide concentration of 0.6 μM and above.

Sequencing:

The amplification products obtained with or without blocking of the DRB3 gene were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Ref. 4303152). The electrophoregrams of the Reverse reaction are given below for the assay without blocking and for the assay with blocking using 0.9 μM of oligonucleotide 5858. AD1

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The montage of the electrophoregrams concerning the region encoding amino acids 56 to 65 (HLA official nomenclature) of the HLA-DRB genes illustrates the

inhibition of the amplification of the HLA-DRB3 gene (Figures 9 and 10).

Sequence expected for	5' > 3'
	56 60 65
DRB1*1301	CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG GAC
DRB3*01	CCT GTC GCC GAG TCC TGG AAC AGC CAG AAG GAC
DRB1*1301+DRB3*01 (forward)	CCT GWY GCC GAG TMC TGG AAC AGC CAG AAG GAC
DRB1*1301+DRB3*01 (reverse)	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG
Sequence read for	
assay without blocking	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG
assay with blocking (0.9 μM)	GGA CTA CGG CTC ATG ACC TTG TCG GTC TTC CTG

Thus, the addition of DRB3-specific blocking primer (oligonucleotide 5858, 3'-C_6-NH_2) inhibits the amplification of the DRB3 gene, as shown by the disappearance of the related bases at positions 57 and 60, demonstrating the absence of the sequence corresponding to the DRB3*01 allele.

DRB3 blocking with 5'-acridine/3'-H oligonucleotide

DNA: OMW line (ECCAC 9058), DRB1*1301, DRB3*01

5' generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1 μM final

3' generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1 μM final

5' blocking HLA-DRB3 primer (oligonucleotide 5967, SEQ ID NO 4): 0, 0.3, 0.6, 0.9, 1.2 μM final

Microplate hybridization:

The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 3 below.

Final conc. of blocking primer 5967 (μM)	Probe 13	Probe 3+6	Probe 52a
0	2356	907	893
0.3	1227	251	5
0.6	1395	239	0
0.9	799	185	4
1.2	965	161	0
0.6/0 ratio	0.60	0.30	0

Table 3: Signals for hybridization of the blocking primer 5967 to various target sequences as a function of its concentration

The calculation of the "value read for 0.6 μM"/"value read without blocking" ratio makes it possible to assess the inhibition of the amplification of the DRB3 gene.

Here again, the addition of oligonucleotide 5967 during amplification inhibits the amplification of the DRB3 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB3 oligonucleotide concentration of 0.3 μM and above.

Example 2: Specific blocking of the amplification of the HLA-DRB4 gene

The situation is comparable to that described in Example 1. In order to simplify the interpretation of the HLA-DRB1 typing, it may be advantageous to limit the HLA-DRB amplification with the generic HLA-DRB primers, to the DRB1 gene, without co-amplification of the DRB4 gene. The present invention describes the use of DRB4-specific blocking primers.

The experimental protocols are identical to those described in Example 1.

DNA: T7526 line (ECCAC 9076), DRB1*-0901, DRB4*01

5′ generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1 μM final

3′ generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1 μM final

5′ blocking HLA-DRB4 primer (oligonucleotide 5965, SEQ ID NO 5): 0, 0.3, 0.6, 0.9, 1.2 μM final

Microplate hybridization:

The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 4 below.

Final conc. of blocking primer 5965 (μM)	Probe 9	Probe 53
0	1769	1320
0.3	1935	110
0.6	1754	41
0.9	1750	29
1.2	1516	14
0.6/0 ratio	0.99	0.03

Table 4: Signals for hybridization of the blocking primer 5965 to various target sequences as a function of its concentration

The calculation of the "value read for 0.6 μM"/"value read without blocking" ratio makes it possible to assess the inhibition of the amplification of the DRB4 gene.

Probe g is specific for the DRB1 gene, and probe 53 is specific for the DRB4 gene. The addition of oligonucleotide 5965 during amplification inhibits the amplification of the DRB4 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB4 oligonucleotide concentration of 0.6 μM and above.

Sequencing:

The amplification products obtained with or without blocking of the DRB4 gene were sequenced using

the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Ref. 4303152). The electrophoregrams of the Reverse reaction are given below for the assay without blocking and for the assay with blocking using 0.9 µM of oligonucleotide 5965. 102

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The montage of the electrophoregrams concerning the region encoding amino acids 29 to 47 (HLA official nomenclature) of the HLA-DRB genes illustrates the inhibition of the amplification of the HLA-DRB4 gene (Figures 11 and 12).

Sequence expected for									5' > 3'										
		30					35					40					45		
DRB1*0901	AGA	GGC	ATC	TAT	AAC	CAA	GAG	GAG	AAC	GTG	CGC	TTC	GAC	AGC	GAC	GTG	GGG	GAG	TAC
DRB4*01	AGA	TAC	ATC	TAT	AAC	CAA	GAG	GAG	TAC	GCG	CGC	TAC	AAC	AGT	GAC	CTG	GGG	GAG	TAC
DRB1*0901+DRB4*01 (forward)	AGA	KRC	ATC	TAT	AAC	CAA	GAG	GAG	WAC	GYG	CGC	TWC	RAC	AGY	GAC	STG	GGG	GAG	TAC
DRB1*0901+DRB4*01 (reverse)	TCT	MYG	TAG	ATA	TTG	GTT	CTC	CTC	WTG	CRC	GCG	AWG	YTG	TCR	CTG	SAC	CCC	CTC	ATG
Sequence read for																			
assay without blocking	TCT	MYG	TAG	ATA	TTG	GTT	CTC	CTC	WTG	CRC	GCG	AWG	YTG	TCR	CTG	SAC	CCC	CTC	ATG
assay with blocking (0.9 µM)	TCT	CCG	TAG	ATA	TTG	GTT	CTC	CTC	TTG	CAC	GCG	AAG	CTG	TCG	CTG	CAC	CCC	CTC	ATG

Thus, the addition of DRB4-specific blocking primer (oligonucleotide 5965, 3'-C_6-NH_2) inhibits the amplification of the DRB4 gene, as shown by the disappearance of the related bases at positions 30, 37, 38, 40, 41, 42 and 44, demonstrating the absence of the sequence corresponding to the DRB4*01 allele.

The present invention can be used for high resolution HLA-DRB1 typing, the specific blocking of the specific amplification of the HLA-DRB3, -DRB4 and -DRB5 genes, and of the HLA-DRB2, -DRB6, -DRB7, -DRB8 and -DRB9 pseudogenes, using blocking primers reducing the analysis to a mixture of one (in the case of a homozygous sample) or of two (in the case of a heterozygous sample) nucleotide sequences. Such a strategy uses HLA-DRB3-specific blocking primers (oligonucleotides 5816 (SEQ ID NO 6), 5868 (SEQ ID NO 7) and 5885 (SEQ ID NO 8), by way of examples), HLA-DRB4-specific primers (oligonucleotides 5883 (SEQ ID NO 9), 5916 (SEQ ID NO 10) and 5917 (SEQ ID NO 11), by way of examples) and HLA-DRB5-

specific primers (oligonucleotides 5021 (SEQ ID NO 12), 5870 (SEQ ID NO 13), 5871 (SEQ ID NO 14), 5881 (SEQ ID NO 15), 5902 (SEQ ID NO 16), 5903 (SEQ ID NO 17), 5913 (SEQ ID NO 18) and 5914 (SEQ ID NO 19), by way of examples), modified at their 3' and 5' ends as described above.

Complete blocking of the HLA-DRB3, -DRB4 and -DRB5 genes can be produced using a mixture of blocking primers.

In Table 5 below, i represents inosine.

bioMérieux reference	SEQ ID NO	Nucleotide sequence (5' > 3')	5' Modif.	3' Modif.
5867	1	ATC CTT CGT GTC CCC ACA GCA CG	-	-
P2	2	TCG CCG CTG CAC TGT GAA G	-	-
5858	3	CCC CCC AGC ACG TTT CTT GGA GCT	-	C_6-NH_2
5967	4	CCC CCC AGC ACG TTT CTT GGA GCT	-acridine	-H
5965	5	CCC ACA GCA CGT TTC TTG GAG CAi GC	-	..C_6-NH_2
5816	6	CCC AGC ACG TTT CTT GGA GCT	-	-
5868	7	CCC CCC AGC ACG TTT CTT GGA GCT	-	-
5885	8	CCC CCC AGC ACG TTT CTT GGA GiT	-	-
5883	9	CAT TTC CTC AAT GGG ACG GAG iiA	-	-
5916	10	CCC CCA GCA CGT TTC TTG GAG CAi GC	-	-
5917	11	CCC ACA GCA CGT TTC TTG GAG CAi GC	-	-
5021	12	CA CGT TTC TTG CAG CAG GA	-	-
5870	13	CA GCA CGT TTC TTG CAG CAG GA	-	-
5871	14	CA iGT TTC TTG CAG CAG GA	-	-
5881	15	CA GCA iGT TTC TTG CAG CAG GA	-	-
5902	16	CCC CCA GCA iGT TTC TTG CAG CAG GA	-	-
5903	17	CCC ACA GCA iGT TTC TTG CAG CAG GA	-	-
5913	18	CCC ACA GCA iGT TTC TTG CAG CAG iA	-	-
5914	19	CCC CCA GCA iGT TTC TTG CAG CAG iA	-	-

Table 5: Nucleotide sequence of the oligonucleotides used as primers for the amplification

Inosine, which is not a natural base, is used in order to weaken the nucleic acid/blocking primer hybrid. Specifically, inosine is linked to its complementary nucleotide via two hydrogen bonds, and therefore, when it is substituted for a pyrimidine, the bonding between the two strands, in its region, is weaker. Since a gene can differ from other related genes by a single base, it is advantageous to substitute, on the blocking primer, which is complementary to the gene, the bases around this essential position with inosines. Since the nucleic acid/blocking primer duplex thus becomes weakened, hybridization can only occur if the primer is perfectly complementary to the target gene sequence. The specificity of the blocking primer is thus reinforced.

The present invention therefore relates to a method for selectively amplifying genes present in a mixture of related genes, using blocking oligonucleotide primers which correspond to oligonucleotides comprising a modified 3′ end which does not allow their elongation during the steps for enzymatically amplifying target genes.

The invention also relates to the use of primers which block in the sense described in claim 1, and which comprise a modified 5′ end which does not allow their displacement or their degradation, in the steps for enzymatically amplifying target genes using a primer specific for a region which is located further toward the 5′ end on the same gene.

In the case of use of primers which block in the sense described above, the modification at the 5′ end is facultative. Thus, two different possibilities exist.

According to a first embodiment, the 3′ -OH group is replaced with a group which is not naturally found in nature, such as an -H, -phosphate, or -dabcyl group, or a carbon-based chain terminated by an $-NH_2$ group, by way of examples.

According to a second embodiment, the 5′ -phosphate group is replaced with a group which is not naturally found in nature, such as a -DMT, acridine or -thiophosphate group, or a "stem-loop" structure, by way of examples.

The blocking primers, as described above, can also comprise modifications of the oligonucleotide in a nonterminal position, and are used in order to promote their hybridization to their target sequences.

The blocking primers, as described above, are capable of hybridizing to the coding strand or to the complementary strand (use of 5′ blocking primers or 3′ blocking primers).

It is also possible to use one blocking primer or a mixture of blocking primers.

The use of blocking primers is particularly advantageous for the methods for amplifying the target sequences, such as for example PCR, TMA or any other technique.

The invention relates finally to the use, for inhibiting the amplification of the HLA-DRB3, -DRB4 and -DRB5 genes, of one or more blocking primers chosen from those which are defined by the sequences SEQ ID os. 3 to 19, and the complementary sequences thereof.